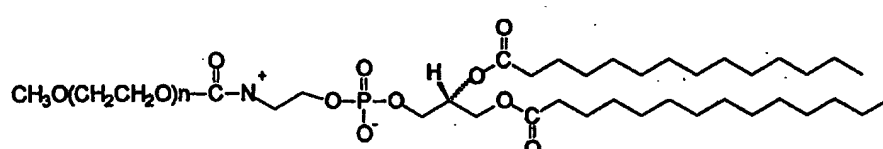# PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 9/127, 48/00, C12N 15/88	A2	(11) International Publication Number: **WO 98/51285**
		(43) International Publication Date: 19 November 1998 (19.11.98)

(21) International Application Number: PCT/US98/09974

(22) International Filing Date: 15 May 1998 (15.05.98)

(30) Priority Data:
60/046,599   15 May 1997 (15.05.97)   US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: CATIONIC AMPHIPHILE FORMULATIONS

(57) Abstract

Novel formulations of cationic amphiphiles are provided that facilitate transport of biologically active (therapeutic) molecules into cells. The formulations comprise one or more cationic amphiphiles, a neutral co–lipid such as diphytanoylphosphatidylethanolamine and one or more derivatives of polyethylene glycol. Therapeutic molecules that can be delivered into cells according to the practice of the invention include DNA, RNA, and polypeptides. Representative uses of the therapeutic compositions of the invention include providing gene therapy, and delivery of antisense polynucleotides or biologically active polypeptides to cells.

Cationic Amphiphile Formulations

This application claims the benefit of U.S. provisional application no. 60/046,599, filed May 15, 1997.

Background of the Invention

The present invention relates to novel formulations of cationic amphiphilic compounds that facilitate the intracellular delivery of biologically active (therapeutic) molecules. The novel formulations of cationic amphiphiles are particularly useful in relation to treating disease states such as by gene therapy.

Effective therapeutic use of many types of biologically active molecules has not been achieved simply because methods are not available to cause delivery of therapeutically effective amounts of such substances into the particular cells of a patient for which treatment therewith would provide therapeutic benefit. Efficient delivery of therapeutically sufficient amounts of such molecules into cells has often proved difficult, if not impossible, since, for example, the cell membrane presents a selectively-permeable barrier. Additionally, even when biologically active molecules successfully enter targeted cells, they may be degraded directly in the cell cytoplasm or even transported to structures in the cell, such as lysosomal compartments, specialized for degradative processes. Thus both the nature of substances that are allowed to enter cells, and the amounts thereof that ultimately arrive at targeted locations within cells, at which they can provide therapeutic benefit, are strictly limited.

Although such selectivity is generally necessary in order that proper cell function can be maintained, it comes with the disadvantage that many

therapeutically valuable substances (or therapeutically effective amounts thereof) are excluded. Additionally, the complex structure, behavior, and environment presented by an intact tissue that is targeted for intracellular delivery of biologically active molecules often interfere substantially with such delivery, in comparison with the case presented by populations of cells cultured *in vitro*.

Examples of biologically active molecules for which effective targeting to a patients' tissues is often not achieved include: (1) numerous proteins including immunoglobin proteins, (2) polynucleotides such as genomic DNA, cDNA, or mRNA (3) antisense polynucleotides; and (4) many low molecular weight compounds, whether synthetic or naturally occurring, such as the peptide hormones and antibiotics.

One of the fundamental challenges now facing medical practitioners is that although the defective genes that are associated with numerous inherited diseases (or that represent disease risk factors including for various cancers) have been isolated and characterized, methods to correct the disease states themselves by providing patients with normal copies of such genes (the technique of gene therapy) are substantially lacking. Accordingly, the development of improved methods of intracellular delivery therefor is of great medical importance.

Examples of diseases that it is hoped can be treated by gene therapy include: inherited disorders such as cystic fibrosis, Gaucher's disease, Fabry's disease, and muscular dystrophy. Representative of acquired disorders that can be treated are: (1) for cancers—multiple myeloma, leukemias, melanomas, ovarian carcinoma and small cell lung cancer; (2) for cardiovascular conditions—progressive heart failure,

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restenosis, and hemophilias; and (3) for neurological conditions—traumatic brain injury.

Gene therapy requires successful transfection of target cells in a patient. Transfection may generally be defined as the process of introducing an expressible polynucleotide (for example a gene, a cDNA, or an mRNA) into a cell. Successful expression of the encoding polynucleotide leads to production in the cells of a normal protein and leads to correction of the disease state associated with the abnormal gene. Therapies based on providing such proteins directly to target cells (protein replacement therapy) are often ineffective for the reasons mentioned above.

Cystic fibrosis, a common lethal genetic disorder, is a particular example of a disease that is a target for gene therapy. The disease is caused by the presence of one or more mutations in the gene that encodes a protein known as cystic fibrosis transmembrane conductance regulator ("CFTR"), and which regulates the movement of ions (and therefore fluid) across the cell membrane of epithelial cells, including lung epithelial cells. Abnormal ion transport in airway cells leads to abnormal mucous secretion, inflammation and infection, tissue damage, and eventually death.

It is widely hoped that gene therapy will provide a long lasting and predictable form of therapy for certain disease states. There remains however a critical need to develop compounds that facilitate entry of functional genes into cells, and whose activity in this regard is sufficient to provide for *in vivo* delivery of genes or other such biologically active therapeutic molecules, preferably in concentrations thereof that are sufficient for intracellular therapeutic effect.

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Reported Developments

Because compounds designed to facilitate intracellular delivery of biologically active molecules must interact with both non-polar and polar environments (in or on, for example, the plasma membrane, tissue fluids, compartments within the cell, and the biologically active molecule itself), such compounds are designed typically to contain both polar and non-polar domains. Compounds having both such domains may be termed amphiphiles, and many lipids and synthetic lipids that have been disclosed for use in facilitating such intracellular delivery (whether for *in vitro* or *in vivo* application) meet this definition. One particularly important class of such amphiphiles is the cationic amphiphiles. In general, cationic amphiphiles have polar groups that are capable of being positively charged at or around physiological pH, and this property is understood in the art to be important in defining how the amphiphiles interact with the many types of biologically active (therapeutic) molecules including, for example, negatively charged polynucleotides such as DNA.

Examples of cationic amphiphilic compounds that have both polar and non-polar domains and that are stated to be useful in relation to intracellular delivery of biologically active molecules are found, for example, in the following references, which contain also useful discussion of (1) the properties of such compounds that are understood in the art as making them suitable for such applications, and (2) the nature of structures, as understood in the art, that are formed by complexing of such amphiphiles with therapeutic molecules intended for intracellular delivery.

(1)   Felgner, et al., <u>Proc. Natl. Acad. Sci. USA</u>, 84, 7413-7417 (1987) disclose use

of positively-charged synthetic cationic lipids including N-[1(2,3-dioleyloxy)propyl]-

N,N,N-trimethylammonium chloride ("DOTMA"), to form lipid/DNA complexes

suitable for transfections. See also Felgner et al., <u>The Journal of Biological</u>

<u>Chemistry</u>, 269(4), 2550-2561 (1994).

(2)   Behr et al., <u>Proc. Natl. Acad. Sci., USA</u> 86, 6982-6986 (1989) disclose

numerous amphiphiles including dioctadecylamidologlycylspermine ("DOGS").

(3)   U.S. Patent 5,283,185 to Epand et al. describes additional classes and

species of amphiphiles including 3β [N-(N¹,N¹ - dimethylaminoethane)-carbamoyl]

cholesterol, termed "DC-chol".

(4)   Additional compounds that facilitate transport of biologically active molecules

into cells are disclosed in U.S. Patent No. 5,264,618 to Felgner et al. See also

Felgner et al., <u>The Journal Of Biological Chemistry</u> 269(4), pp. 2550-2561 (1994) for

disclosure therein of further compounds including "DMRIE" 1,2-dimyristyloxypropyl-

3-dimethyl-hydroxyethyl ammonium bromide, which is discussed below.

(5)   Reference to amphiphiles suitable for intracellular delivery of biologically

active molecules is also found in U.S. Patent No. 5,334,761 to Gebeyehu et al., and

in Felgner et al., <u>Methods</u> (Methods in Enzymology), 5, 67-75 (1993).

Although the compounds mentioned in the above-identified references have

been demonstrated to facilitate (although in many such cases only *in vitro*) the entry

of biologically active molecules into cells, it is believed that the uptake efficiencies

provided thereby are insufficient to support numerous therapeutic applications,

particularly gene therapy. Additionally, since the above-identified compounds are

understood to have only modest activity, substantial quantities thereof must be used leading to concerns about the toxicity of such compounds or of the metabolites thereof. Accordingly there is a need to develop a "second generation" of cationic amphiphiles with enhanced activity that will preferably lead to successful therapies. Such a second generation of cationic amphiphiles is described, for example, in published international patent specification WO 96/18372 entitled Cationic Amphiphiles and Plasmids for Intracellular Delivery of Therapeutic Molecules, internationally published on June 20, 1996. The WO 96/18372 publication also discloses formulations of cationic amphiphiles of relevance to the practice of the present invention.

Summary of the Invention

This invention provides for formulations of cationic amphiphiles that are particularly effective to facilitate transport of biologically active molecules, such as a polynucleotide, into cells. The formulations comprise a derivative of polyethylene glycol (PEG), and one or more cationic amphiphiles, and optionally a neutral co-lipid. As defined herein, a derivative of PEG is any hydrophobic group attached to the PEG polymer. Representative of suitable PEG derivatives are polyethylene glycol 5000—dimyristoylphosphatidylethanolamine (hereinafter $PEG_{(5000)}$—DMPE), and polyethylene glycol 2000—dimyristoylphosphatidylethanolamine (hereinafter $PEG_{(2000)}$—DMPE). In a typical case, the formulation is then mixed with a biologically active molecule such as a polynucleotide, to form a therapeutic composition in which the cationic amphiphile and polynucleotide are in intimate contact. Without being

limited as to theory, it is believed that the PEG derivative stabilizes the therapeutic

composition by preventing undesired further aggregation of the cationic

amphiphile/polynucleotide complexes. Important benefits, preferably clinical

benefits, which result from use of PEG-derivatives in such compositions, preferably

therapeutic compositions, include: (1) cationic amphiphile/polynucleotide complexes

may be maintained at higher concentrations in solution, and (2) such compositions

may be more efficiently delivered by aerosol to the lung.

Representative of the neutral co-lipids that are useful in the practice of the

invention are diphytanoylphosphatidylethanolamine and dioleoyl

phosphatidylethanolamine ("DOPE"). Use of diphytanoylphosphatidylethanolamine

is preferred according to the practice of the present invention.

Representative of cationic amphiphiles that are useful in the practice of the
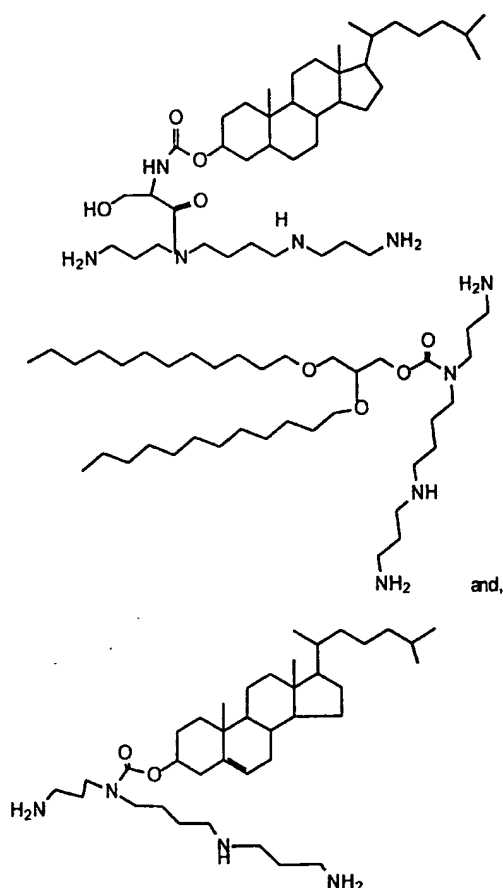
invention are:

and other amphiphiles as are known in the art including those described in
international patent publication WO 96/18372.

In a further aspect, the invention provides a method for facilitating the transfer
of biologically active molecules into cells comprising the steps of: preparing a
dispersion of one or more cationic amphiphiles, a neutral co-lipid, and a PEG -
derivative; mixing said dispersion with a biologically active molecule to form a

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complex between said amphiphile and said biologically active molecule; and

contacting cells with said complex, thereby facilitating transfer of said biologically-

active molecule into the cells.

Further additional and representative aspects of the invention are described

according to the Detailed Description of the Invention which follows directly.

Brief Description of the Drawing

Figure 1 depicts a representative structure for PEG-DMPE, a PEG derivative

useful in the practice of the invention.

Detailed Description of the Invention

According to the practice of the invention, a derivative of polyethylene glycol

is used to stabilize, and to enhance the transfecting properties of, therapeutic

compositions than comprise a cationic amphiphile. According to the practice of the

present invention it has been surprisingly determined that the stability and

transfection-enhancing capability of cationic amphiphile/neutral co-lipid compositions

can be substantially improved by adding to such formulations small additional

amounts of one or more derivatized polyethylene glycol compounds. Such

enhanced performance is particularly apparent whether measured by stability of

cationic amphiphile/co-lipid formulations to storage and manipulation, including in

liquid (suspended) form, and during aerosol delivery of such formulations when

containing a therapeutic molecule, particularly polynucleotides.

Derivatives of polyethylene glycol useful in the practice of the invention

include numerous phospholipid conjugates of polyethylene glycol. With respect to

the design of such derivatives, selection of certain phospholipids is preferred, as is

the selection of particular polyethylene glycol polymers. The structure of a typical

derivative is provided in Figure 1.

With respect to selection of the phospholipid for inclusion therein, species of

phosphatidylethanolamine are preferred. It is preferred that the fatty acid(acyl)

chains thereof be selected from the group consisting of C_{10}, C_{12}, and C_{14}, most

preferably the fully saturated species. Each phospholipid contains 2 fatty acid

chains, and it is within the practice of the invention to provide as phospholipid

component, species having 2 different acyl chains. Two highly preferred species

thereof include dimyristoylphosphatidylethanolamine (di C_{14}) ("DMPE") and

dilaurylphosphatidylethanolamine (di C_{12}) ("DLPE"). Use of

dipalmitoylphosphatidylethanolamine (di C_{16}) ("DPPE") and

distearoylphosphatidylethanolamine (di C_{18}) ("DSPE") in order to derivatize the PEG

polymer is not preferred according to the practice of the invention.

With respect to selection of the PEG polymer, it is preferred that the polymer

be linear, having a molecular weight from about 1,000 and 10,000, preferred species

thereof including those having molecular weights from about 1500 to 7000, with

2000 and 5000 being examples of useful, and commercially available sizes. In the

practice of the invention, it is convenient to use derivatized PEG species provided

from commercial sources, and it is noted that the molecular weight assigned to PEG

in such products often represents a molecular weight average, there being shorter

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and longer molecules in the product. Such molecular weight ranges are typically a consequence of the synthetic procedures used, and the use of any such product is within the practice of the invention as long as a substantial fraction of polymer population falls within or near the above-suggested MW range of 1000-10,000.

It is also within the practice of the invention to use derivatized-PEG species that (1) include more than one attached phospholipid, or (2) include branched PEG sequence, or (3) include both of modifications (1) and (2).

Accordingly, preferred species of derivatized PEG include

(a)   polyethylene glycol 5000-dimyristoylphosphatidylethanolamine, also referred to as $PEG_{(5000)}$—DMPE;

(b)   polyethylene glycol 2000-dimyristoylphosphatidylethanolamine, also referred to as $PEG_{(2000)}$—DMPE);

(c)   polyethylene glycol 5000-dilaurylphosphatidylethanolamine, also referred to as $PEG_{(5000)}$—DLPE); and

(d)   polyethylene glycol 2000-dilaurylphosphatidylethanolamine, also referred to as $PEG_{(2000)}$—DLPE).

Certain phospholipid derivatives of PEG may be obtained from commercial suppliers. For example, the following species: di C14:0, di C16:0, di C18:0, di C18:1, and 16:0/18:1 are available as average 2000 or average 5000 MW PEG derivatives from Avanti Polar Lipids, Alabaster, AL, USA, as catalog nos. 880150, 880160, 880120, 880130, 880140, 880210, 880200, 880220, 880230, and 880240.

Selection of Neutral Co-lipids

Formulating neutral co-lipids with cationic amphiphiles substantially enhances transfection capability. Representative neutral co-lipids include dioleoylphosphatidylethanolamine ("DOPE"), the species most commonly used in the art, diphytanoylphosphatidylethanolamine, lyso-phosphatidylethanolamines other phosphatidyl-ethanolamines, phosphatidylcholines, lyso-phosphatidylcholines and cholesterol. Typically, a preferred molar ratio of cationic amphiphile to colipid is about 1:1. However, it is within the practice of the invention to vary this ratio (see Example 3 below), including also over a considerable range, although a ratio from 2:1 through 1:2 is usually preferable. Use of diphytanoylphosphatidylethanolamine is highly preferred according to the practice of the present invention, as is use of "DOPE".

Preferred Molar Ratios of Formulation Ingredients

According to the practice of the invention, preferred formulations may also be defined in relation to mole percent of PEG derivative. For example, with respect to a formulation of cationic amphiphile/neutral co-lipid/PEG derivative that is 1:2: 0.05 on a molar basis, the derivative is present at about 0.05/3.05 x100 or 1.6 mole percent. According to the practice of the invention, use of formulations ranging from about 0.1 to 10 mole percent of PEG-derivative is preferred, with formulations of about 0.5 to 5.0 mole percent generally being most preferred.

A representative preferred formulation according to the practice of the present invention has a cationic amphiphile : neutral colipid: PEG-derivative molar

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composition ratio of about 1:2:0.125. Another representative formulation has a

cationic amphiphile : neutral co-lipid : PEG-derivative molar composition ratio of

about 1:2:0.5.

In preferred examples thereof, the neutral co-lipid is

diphytanoylphosphatidylethanolamine, or is DOPE, and the PEG derivative is a

DMPE or DLPE conjugate of PEG_{2000} or PEG_{5000}. In a highly preferred example, the

neutral co-lipid is diphytanoylphosphatidyl ethanolamine, and the PEG derivative is

$PEG_{(2000)}$-DMPE, wherein the molar ratios of said composition are 1 (amphiphile): 2

(neutral co-lipid) : 0.125 (PEG-derivative).

As aforementioned, the capability of a wide variety of cationic amphiphiles to

facilitate transport of biologically active molecules, particularly polynucleotides, into

cells, can be enhanced according to the teachings of the present invention.

Representative of such amphiphiles are (see the WO 96/18372 publication):
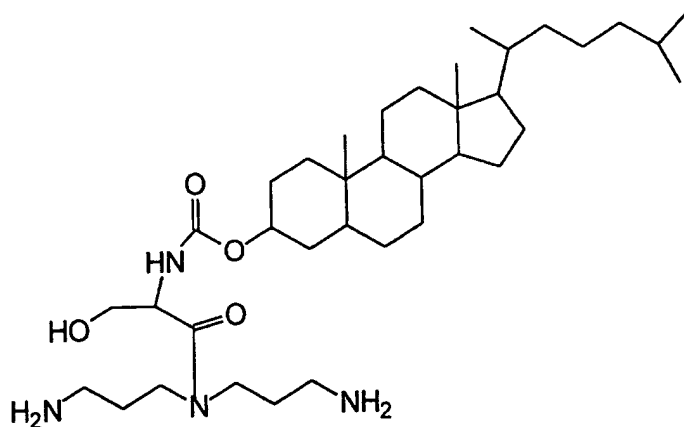
having the IUPAC name {1-[(4-Amino-butyl)-(3-amino-propyl)-carbamoyl]-2-hydroxy-

ethyl)-carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-

2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H* -cyclopenta[a]phenanthren-
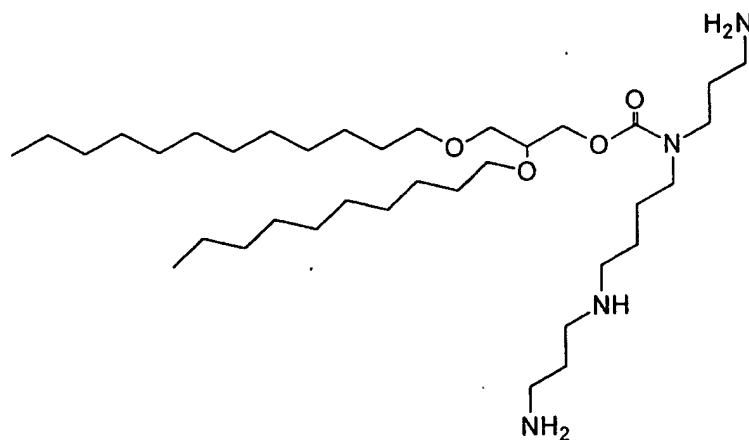
3-yl ester;

({(3-Amino-propyl)-[4-(3-amino-propylamino)-butyl]-carbamoyl}-methyl)-carbamic

acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-

tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl ester;

amphiphile No. 67, having the IUPAC name (3-Amino-propyl)-[4-(3-amino-

propylamino)-butyl]-carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-

2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-

3-yl ester; and

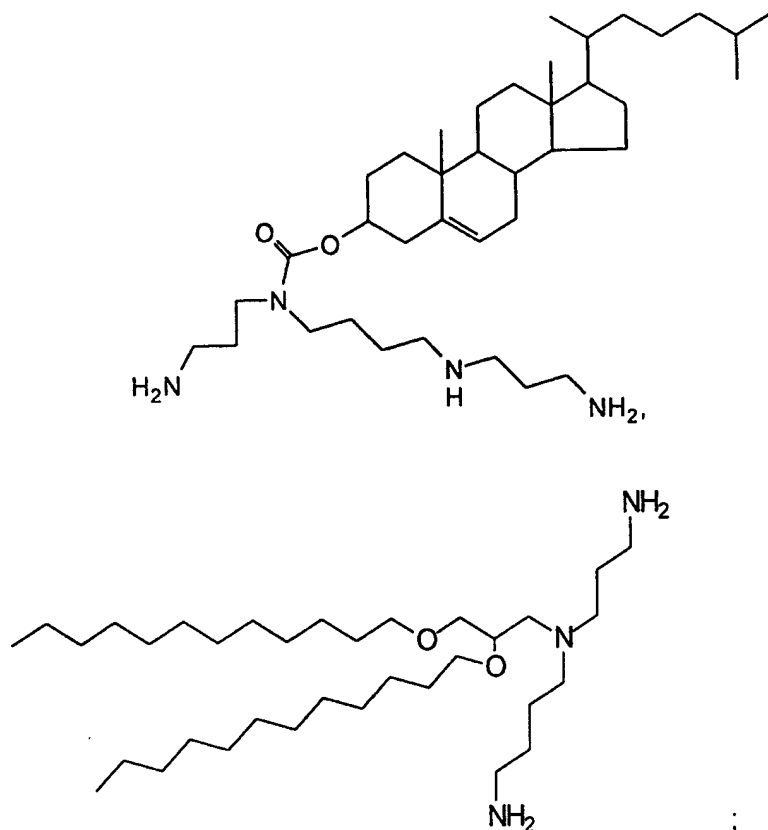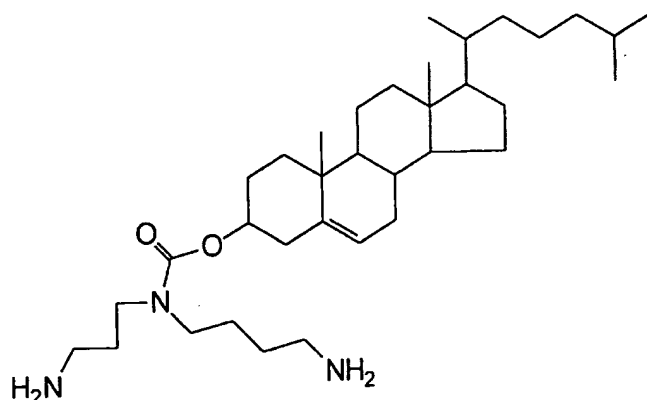amphiphile No. 53, having the IUPAC name (4-Amino-butyl)-(3-amino-propyl)-

carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-

2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-

3-yl ester.

Transacylation Reactions

Although heretofore unrecognized in the art, it has been determined also that

certain co-lipids may react chemically with certain types of cationic amphiphiles

under conditions of co-storage, there resulting new molecular species. Generation

of such new species is believed to occur via mechanisms such as transacylation.

For a further discussion thereof, see international patent publication WO 96/18372 at

pages 43-44, and also Figure 4 thereof.

It is to be understood that therapeutically-effective pharmaceutical compositions of the present invention may or may not contain such transacylation byproducts, or other byproducts, and that the presence of such byproducts does not prevent the therapeutic use of the compositions containing them. Rather use of such compositions is within the practice of the invention, and such compositions and the novel molecular species thereof are therefore specifically claimed.

Preparation of Pharmaceutical Compositions and Administration Thereof

The present invention provides for pharmaceutical compositions that facilitate intracellular delivery of therapeutically effective amounts of biologically active molecules. Pharmaceutical compositions of the invention facilitate entry of biologically active molecules into tissues and organs such as the gastric mucosa, heart, lung, and solid tumors. Additionally, compositions of the invention facilitate entry of biologically active molecules into cells that are maintained in vitro, such as in tissue culture. The amphiphilic nature of the compounds of the invention enables them to associate with the lipids of cell membranes, other cell surface molecules, and tissue surfaces, and to fuse or to attach thereto. One type of structure that can be formed by amphiphiles is the liposome, a vesicle formed into a more or less spherical bilayer, that is stable in biological fluids and can entrap biological molecules targeted for intracellular delivery. By fusing with cell membranes, such liposomal compositions permit biologically active molecules carried therewith to gain access to the interior of a cell through one or more cell processes including endocytosis and pinocytosis. However, unlike the case for many classes of

amphiphiles or other lipid-like molecules that have been proposed for use in therapeutic compositions, the cationic amphiphiles of the invention need not form highly organized vesicles in order to be effective, and in fact can assume (with the biologically active molecules to which they bind) a wide variety of loosely organized structures. Any of such structures can be present in pharmaceutical preparations of the invention and can contribute to the effectiveness thereof.

Biologically active molecules that can be provided intracellularly in therapeutic amounts using the amphiphiles of the invention include: (a) polynucleotides such as genomic DNA, cDNA, and mRNA that encode for therapeutically useful proteins as are known in the art,

(b) ribosomal RNA;

(c) antisense polynucleotides, whether RNA or DNA, that are useful to inactivate transcription products of genes and which are useful, for example, as therapies to regulate the growth of malignant cells; and

(d) ribozymes.

In general, and owing to the potential for leakage of contents therefrom, vesicles or other structures formed from numerous of the cationic amphiphiles are not preferred by those skilled in the art in order to deliver low molecular weight biologically active molecules. Although not a preferred embodiment of the present invention, it is nonetheless within the practice of the invention to deliver such low molecular weight molecules intracellularly. Representative of the types of low molecular weight biologically active molecules that can be delivered include hormones and antibiotics.

Cationic amphiphile species of the invention may be blended so that two or more species thereof are used, in combination, to facilitate entry of biologically active molecules into target cells and/or into subcellular compartments thereof. Cationic amphiphiles of the invention can also be blended for such use with amphiphiles that are known in the art.

Dosages of the pharmaceutical compositions of the invention will vary, depending on factors such as half-life of the biologically-active molecule, potency of the biologically-active molecule, half-life of the amphiphile(s), any potential adverse effects of the amphiphile(s) or of degradation products thereof, the route of administration, the condition of the patient, and the like. Such factors are capable of determination by those skilled in the art.

A variety of methods of administration may be used to provide highly accurate dosages of the pharmaceutical compositions of the invention. Such preparations can be administered orally, parenterally, topically, transmucosally, or by injection of a preparation into a body cavity of the patient, or by using a sustained-release formulation containing a biodegradable material, or by onsite delivery using additional micelles, gels and liposomes. Nebulizing devices, powder inhalers, and aerosolized solutions are representative of methods that may be used to administer such preparations to the respiratory tract.

Additionally, the therapeutic compositions of the invention can in general be formulated with excipients (such as the carbohydrates lactose, trehalose, sucrose, mannitol, maltose or galactose, and inorganic or organic salts) and may also be lyophilized (and then rehydrated) in the presence of such excipients prior to use.

Conditions of optimized formulation for each amphiphile of the invention are capable

of determination by those skilled in the pharmaceutical art.

Accordingly, a principal aspect of the invention involves providing a

composition that comprises a biologically active molecule (for example, a

polynucleotide) and one or more cationic amphiphiles (including optionally one or

more co-lipids), and then maintaining said composition in the presence of one or

more excipients as aforementioned, said resultant composition being in liquid or

solid (preferably lyophilized) form, so that: (1) the therapeutic activity of the

biologically active molecules is substantially preserved; (2) the transfection-

enhancing nature of the amphiphile (or of amphiphile/DNA complex) is maintained.

Without being limited as to theory, it is believed that the excipients stabilize the

interaction of the amphiphile and biologically active molecule through one or more

effects including:

(1)   minimizing interactions with container surfaces,

(2)   preventing irreversible aggregation of the complexes, and

(3)   maintaining amphiphile/DNA complexes in a chemically-stable state, i.e.,

preventing oxidation and/or hydrolysis.

Although the presence of excipients in the pharmaceutical compositions of

the invention stabilizes the compositions and facilitates storage and manipulation

thereof, it has also been determined that moderate concentrations of numerous

excipients may interfere with the transfection-enhancing capability of pharmaceutical

formulations containing them. In this regard, an additional and valuable

characteristic of the amphiphiles of the invention is that any such potentially adverse

effect can be minimized owing to the greatly enhanced *in vivo* activity of the

amphiphiles of the invention in comparison with amphiphilic compounds known in

the art. Without being limited as to theory, it is believed that osmotic stress (at low

total solute concentration) may contribute positively to the successful transfection of

polynucleotides into cells *in vivo*. Such a stress may occur when the pharmaceutical

composition, provided in unbuffered water, contacts the target cells. Use of such

otherwise preferred compositions may therefore be incompatible with treating target

tissues that already are stressed, such as has damaged lung tissue of a cystic

fibrosis patient. Accordingly, and using sucrose as an example, selection of

concentrations of this excipient that range from about 15 mM to about 200 mM

provide a compromise between the goals of (1) stabilizing the pharmaceutical

composition to storage and (2) minimizing any effects that high concentrations of

solutes in the composition may have on transfection performance.

Selection of optimum concentrations of particular excipients for particular

formulations is subject to experimentation, but can be determined by those skilled in

the art for each such formulation.

An additional aspect of the invention concerns the protonation state of the

cationic amphiphiles of the invention prior to their contacting plasmid DNA in order to

form a therapeutic composition, or prior to the time when said therapeutic

composition contacts a biological fluid. It is within the practice of the invention to

provide fully protonated, partially protonated, or free base forms of the amphiphiles

in order to form, or maintain, such therapeutic compositions.

Examples

The following Examples are representative of the practice of the invention. In general, assay procedures and other methodology applicable to the practice of the present invention are described in international patent publication WO 96/18372, published on June 20, 1996 to which the reader is directed.

Example 1 - Cell Transfection Assay

Separate 3.35 μmole samples of amphiphile No. 53 and the neutral lipid dioleoylphosphatidylethanolamine ("DOPE") were each dissolved in chloroform as stock preparations. Following combination of the solutions (as a 1:1 molar composition), a thin film was produced by removing chloroform from the mixture by evaporation under reduced pressure (20 mm Hg). The film was further dried under vacuum (1 mm Hg) for 24 hours. As aforementioned, some of the amphiphiles of the invention participate in transacylation reactions with co-lipids such as DOPE, or are subject to other reactions which may cause decomposition thereof. Accordingly, it is preferred that amphiphile/co-lipid compositions be stored at low temperature, such as -70 degrees C under inert gas, until use.

To produce a dispersed suspension, the lipid film was then hydrated with sterile deionized water (1 ml) for 10 minutes, and then vortexed for 2 minutes (sonication for 10 to 20 seconds in a bath sonicator may also be used, and sonication has proved useful for other amphiphiles such as DC-chol). The resulting suspension was then diluted with 4 ml of water to yield a solution that is 670 μM in cationic amphiphile and 670 μM in neutral colipid.

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Similar experiments were also performed using other amphiphiles of the invention. With respect to amphiphile No. 67, the optimum molar ratio of amphiphile to DOPE under the conditions tested was determined to be 1:2, not 1:1. Optimized ratios for any of the amphiphiles of the invention can be determined by following, generally, the procedures described herein.

For preparation of the transfecting solution, DNA encoding for β-galactosidase (pCMVβ, ClonTech., Palo Alto, CA) was dissolved in OptiMEM culture medium (Gibco/ BRL No. 31885-013). The resulting solution had a DNA concentration of 960 μM (assuming an average molecular weight of 330 daltons for nucleotides in the encoding DNA). The construct pCF1-β (described below) may also be used and generally provides about a 2-fold enhancement over pCMVβ.

The following procedure was used to test a 1:1 molar mixture of the cationic amphiphile No. 53 in combination with DOPE. A 165 μl aliquot of amphiphile No. 53 (670 μM) containing also the colipid (at 670 μM) was pipetted into 8 separate wells in a 96-well plate containing OptiMEM (165 μl) in each well. The resulting 335 μM solutions were then serially diluted 7 times to generate 8 separate amphiphile-containing solutions having concentrations ranging from 335 μM to 2.63 μM, with each resultant solution having a volume of 165 μl. Thus, 64 solutions were prepared in all, there being 8 wells each of 8 different concentrations of amphiphile/DOPE.

Independently, DNA solutions (165 μl, 960 μM) were pipetted into 8 wells containing OptiMEM (165 μl), and the resulting 480 μM solutions were then serially diluted 7 times to generate 8 separate 165 μl solutions from each well, with the concentrations of DNA in the wells ranging from 480 μM to 3.75 μM.

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The 64 test solutions (cationic amphiphile: neutral lipid) were then combined

with the 64 DNA solutions to give separate mixtures in 64 wells, each having a

volume of 330µl, with DNA concentrations ranging from 240 µM to 1.875 µM along

one axis, and lipid concentrations ranging from 167 µM to 1.32 µM along the other

axis. Thus 64 solutions were prepared in all, each having a different amphiphile:

DNA ratio and/or concentration. The solutions of DNA and amphiphile were allowed

to stand for 15 to 30 minutes in order to allow complex formation.

A CFT-1 cell line (human cystic fibrosis bronchial epithelial cells immortalized

with transforming proteins from papillomavirus) provided by Dr. James Yankaskas,

University of North Carolina, Chapel Hill, was used for the *in vitro* assay. The cells

are homozygous for a mutant allele (deletion of phenylalanine at position 508,

hereinafter Δ F508) of the gene encoding for cystic fibrosis transmembrane

conductance regulator ("CFTR") protein. CFTR is a cAMP-regulated chloride (Cl$^-$)

channel protein. Mutation of the CFTR gene results typically in complete loss (or at

least substantial impairment) of Cl$^-$ channel activity across, for example, cell

membranes of affected epithelial tissues.

The Δ F508 mutation is the most common mutation associated with cystic

fibrosis disease. For a discussion of the properties of the Δ F508 mutation and the

genetics of cystic fibrosis disease see, in particular, Cheng et al., Cell, 63, 827-834

(1990). See also Riordan et al., Science, 245, 1066-1073 (1989); published

European Patent Application No. 91301819.8 of Gregory et al., bearing publication

number 0 446 017 A1; and Gregory et al., Nature, 347, 382-385 (1990).

The cells were cultured in Hams F12 nutrient media (Gibco/BRL No. 31765-027) supplemented with 2% fetal bovine serum ("FBS", Irvine Scientific, No. 3000) and 7 additional supplements. Cells were then plated into 96-well tissue culture plates at a density of approximately 7,500 cells/well. Before being used in the assay, cells were allowed to grow for periods of 5-7 days until a confluent pattern had been achieved.

Following the allotted time period, three 96-well plates with CFT-1 cells were aspirated in order to remove the growth medium. The various concentrations of DNA-lipid complex (in 100 µl aliquots) were transferred to each of three 96-well plates bringing the DNA-lipid complexes in contact with the cells. DNA-only/cell and lipid-only/cell control wells were also prepared on one of the three plates.

The 100 µl solutions of DNA-lipid complex were maintained over the cells for 6 hours, after which 50 µl of 30% FBS (in OptiMEM) was added to each well. After a further 20-hour incubation period, an additional 100 µl of 10% FBS in OptiMEM was also added. Following a further 24-hour incubation period, cells were assayed for expression of protein and β-galactosidase.

For the assays, the resultant medium was removed from the plates and the cells washed with phosphate buffered saline. Lysis buffer (50 µl, 250 mM Tris-HCl, pH 8.0, 0.15% Triton X-100) was then added, and the cells were lysed for 30 minutes. The 96-well plates were carefully vortexed for 10 seconds to dislodge the cells and cell debris, and 5 µl volumes of lysate from each well were transferred to a plate containing 100 µl volumes of Coomassie Plus® protein assay reagent (Pierce Company, No. 23236). The protein assay plates were read by a Bio-Rad Model 450

plate-reader containing a 595 nm filter, with a protein standard curve included in every assay.

The level of β-galactosidase activity in each well was measured by adding phosphate buffered saline (50 μl) to the remaining lysates, followed by addition of a buffered solution consisting of chlorophenol red galactopyranoside (100 μl, 1 mg per ml, Calbiochem No. 220588), 60 mM disodium hydrogen phosphate pH 8.0, 1 mM magnesium sulfate, 10 mM potassium chloride, and optionally 50 mM 2-mercaptoethanol. The chlorophenol red galactopyranoside, following enzymatic (β-galactosidase) hydrolysis, gave a red color which was detected by a plate-reader containing a 570 nm filter. A β-galactosidase (Sigma No. G6512) standard curve was included to calibrate every assay.

Following subtraction of background readings, optical data determined by the plate-reader allowed determination of β-galactosidase activity and protein content. In comparison to the amount of β-galactosidase expressed by known transfectants, for example, DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide), compounds of the invention are particularly effective in transfecting airway epithelial cells and inducing therein β-galactosidase expression. Relative to DMRIE:DOPE (1:1), the amphiphile 53:DOPE mixture (at 1:1) demonstrated improved transfection efficiency.

Example 2 - CAT Assay

This assay was used to assess the ability of the cationic amphiphiles of the invention to transfect cells in vivo from live specimens. In the assay, the lungs of

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balb/c mice were instilled intra-nasally (the procedure can also be performed trans-

tracheally) with 100 µl of cationic amphiphile No. 53:DNA complex, which was

allowed to form during a 15-minute period prior to administration according to the

following procedure. The amphiphile (premixed with co-lipid, see below) was

hydrated in water for 10 minutes, a period sufficient to yield a suspension at twice

the final concentration required. This was vortexed for two minutes and aliquoted to

provide 55 microliter quantities for each mouse to be instilled. Similarly, DNA

encoding the reporter (CAT) gene was diluted with water to a concentration twice the

required final concentration, and then aliquoted at 55 microliters for each mouse to

be instilled. The lipid was gently combined with the DNA (in a polystyrene tube), and

the complex allowed to form for 15 minutes before the mice were instilled therewith

(the lipid and DNA are both warmed to 30°C for 5 minutes prior to mixing and

maintained at 30°C during the 15 minutes of complex formation to reduce the

likelihood of complex precipitation).

The plasmid used, pCF1/CAT (see Example 4, pages 82-85, and Figure 18A

of international patent publication WO 96/18372 published June 20, 1996), provides

an encoding DNA for chloramphenicol acetyl transferase enzyme.

Two days following transfection, mice were sacrificed, and the lungs and

trachea removed, weighed, and homogenized in a buffer solution (250 mM Tris, pH

7.8, 5mM EDTA). The homogenate was clarified by centrifugation, and the

deacetylases therein were inactivated by heat treatment at 65°C for twenty minutes.

Lysate was incubated for thirty minutes with acetyl coenzyme A and C^{14-}

chloramphenicol (optimum times vary somewhat for the different amphiphile species

of the invention). CAT enzyme activity was then visualized by thin layer chromatography ("TLC") following an ethyl acetate extraction. Enzyme activity was quantitated by comparison with a CAT standard curve.

The presence of the enzyme CAT will cause an acetyl group to be transferred from acetylcoenzyme A to C^{14}--chloramphenicol. The acetylated/radiolabeled chloramphenicol migrates faster on a TLC plate and thus its presence can be detected. The amount of CAT that had been necessary to generate the determined amount of acetylated chloramphenicol can then be calculated from standards.

The activity of amphiphile No.53 was determined in the CAT assay in relation to the recognized transfection reagents DMRIE and DC-Chol. Enhanced ability of the No. 53 amphiphile (measured as ng CAT activity per 100 mg lung tissue) to transfect cells in vivo was determined in relation to DMRIE.

For the cationic amphiphiles of the invention, optimized compositions for in vivo testing were extrapolated from in vitro results. This facilitated the screening of large numbers of amphiphiles and produced broadly, if not precisely, comparable data. Thus, the ratio, for in vivo testing, of amphiphile concentration to DOPE concentration, was taken from the in vitro experiments, as was the optimized ratio of amphiphile concentration to DNA concentration (see Example 1). Accordingly, for such amphiphiles the in vivo test concentration was fixed at 1mM, thereby fixing also the co-lipid concentration. [Broadly, the molar ratio of the amphiphile to co-lipid DOPE ranged from 1:2 through 1:1 to (about) 2:1]. The concentration of plasmid DNA varied for each amphiphile species tested in order to duplicate the optimized amphiphile/DNA ratio that had been determined in vitro.

Example 3- Construction of vectors

As aforementioned, numerous types of biologically active molecules can be transported into cells in therapeutic compositions that comprise one or more of the cationic amphiphiles of the invention. In an important embodiment of the invention, the biologically active macromolecule is an encoding DNA. There follows a description of novel vectors (plasmids) that are preferred in order to facilitate expression of such encoding DNAs in target cells.

Construction of pCFl

A map of pCF1/CAT is shown in Figure 18, panel A, of aforementioned international patent publication WO 96/18372.

Briefly, pCF1 contains the enhancer/promoter region from the immediate early gene of cytomegalovirus (CMV). A hybrid intron is located between the promoter and the transgene cDNA. The polyadenylation signal of the bovine growth hormone gene was selected for placement downstream from the transgene. The vector also contains a drug-resistance marker that encodes the aminoglycosidase 3'-phosphotransferase gene (derived from the transposon Tn903, A. Oka et al., Journal of Molecular Biology, 147, 217-226, 1981) thereby conferring resistance to kanamycin. Further details of pCF1 structure are provided directly below, including description of placement therein of a cDNA sequence encoding for cystic fibrosis transmembrane conductance regulator (CFTR) protein.

The pCF1 vector is based on the commercially available vector pCMVβ (Clontech). The pCMVβ construct has a pUC19 backbone (J. Vieira, et al., Gene,

19, 259-268, 1982) that includes a prokaryotic origin of replication derived originally from pBR322.

Basic features of the pCFI-plasmid (as constructed to include a nucleotide sequence coding for CFTR) are as follows. Proceeding clockwise the human cytomegalovirus immediate early gene promoter and enhancer, a fused tripartite leader from adenovirus and a hybrid intron, a linker sequence, the CFTR cDNA, an additional linker sequence, the bovine growth hormone polyadenylation signal, pUC origin of replication and backbone, and the kanamycin resistance gene. The pCF1-CFTR plasmid has been completely sequenced on both strands.

The human cytomegalovirus immediate early gene promoter and enhancer spans the region from nucleotides 1-639. This corresponds to the region from -522 to +72 relative to the transcriptional start site (+1) and includes almost the entire enhancer region from -524 to -118 as originally defined by Boshart et al., Cell, 41, 521-530 (1985). The CAAT box is located at nucleotides 486-490 and the TATA box is at nucleotides 521-525 in pCF1-CFTR. The CFTR transcript is predicted to initiate at nucleotide 548, which is the transcriptional start site of the CMV promoter.

The hybrid intron is composed of a fused tri-partite leader from adenovirus containing a 5' splice donor signal, and a 3' splice acceptor signal derived from an IgG gene. The elements in the intron are as follows: the first leader (nucleotides 705-745), the second leader (nucleotides 746-816), the third leader (partial, nucleotides 817-877), the splice donor sequence and intron region from the first leader (nucleotides 878-1042), and the mouse immunoglobulin gene splice donor sequence (nucleotides 1043-1138). The donor site (G | GT) is at nucleotides 887-

888, the acceptor site (<u>AG</u> | G) is at nucleotides 1128-1129 , and the length of the intron is 230 nucleotides. The CFTR coding region comprises nucleotides 1183-5622.

Within the CFTR-encoding cDNA of pCF1-CFTR, there are two differences from the originally-published predicted cDNA sequence (J. Riordan et al., <u>Science</u>, 245, 1066-1073, 1989); (1) an A to C change at position 1990 of the CFTR cDNA which corrects an error in the original published sequence, and (2) a T to C change introduced at position 936. The change at position 936 was introduced by site-directed mutagenesis and is silent but greatly increases the stability of the cDNA when propagated in bacterial plasmids (R. J. Gregory et al., <u>Nature</u>, 347, 382-386, 1990). The 3' untranslated region of the predicted CFTR transcript comprises 51 nucleotides of the 3' untranslated region of the CFTR cDNA, 21 nucleotides of linker sequence and 114 nucleotides of the BGH poly A signal.

The BGH poly A signal contains 90 nucleotides of flanking sequence 5' to the conserved AAUAAA and 129 nucleotides of flanking sequence 3' to the AAUAAA motif. The primary CFTR transcript is predicted to be cleaved downstream of the BGH polyadenylation signal at nucleotide 5808. There is a deletion in pCF1-CFTR at position +46 relative to the cleavage site, but the deletion is not predicted to effect either polyadenylation efficiency or cleavage site accuracy, based on the studies of E.C. Goodwin et al., <u>J. Biol. Chem.</u>, 267, 16330-16334 (1992). After the addition of a poly A tail, the size of the resulting transcript is approximately 5.1 kb.

Example 4-Correction of Chloride Ion Transport Defect in Airway Epithelial Cells of a

Cystic Fibrosis Patient by Cationic Amphiphile-Mediated Gene Transfer

A recommended procedure for formulating and using the pharmaceutical

compositions of the invention to treat cystic fibrosis in human patients is as follows.

Following generally the procedures described in Example 1, a thin film

(evaporated from chloroform) can be produced wherein amphiphile No. 53 and

DOPE are present in the molar ratio of 1:1 [Alternatively, the chloroform can be

removed in a dessicator placed in a cooling bath at approximately -10 degrees C

under high vacuum, for example 1 mm Hg, for 12-24 hours]. The amphiphile-

containing film is then rehydrated in water-for -injection with gentle vortexing to a

resultant amphiphile concentration of about 3mM. However, in order to increase the

amount of amphiphile/DNA complex that may be stably delivered by aerosol as a

homogeneous phase (for example, using a Puritan Bennett Raindrop nebulizer from

Lenexa Medical Division, Lenexa, KS, or the PARI LC Jet™ nebulizer from PARI

Respiratory Equipment, Inc., Richmond, VA), it may be advantageous to prepare the

amphiphile-containing film to include also one or more further ingredients that act to

stabilize the final amphiphile/DNA composition. Accordingly, it may be preferred to

prepare the amphiphile-containing film using an additional ingredient, $PEG_{(5000)}$-

DMPE. [A suitable source of PEG-DMPE, polyethylene glycol 5000-

dimyristoylphoshatidyl ethanolamine, is Catalog No. 880210 from Avanti Polar

Lipids, Alabaster, AL]. Additional fatty acid species of PEG-PE may be used in

replacement therefor.

Without being limited as to theory, $PEG_{(5000)}$-DMPE is believed to stabilize the therapeutic compositions by preventing further aggregation of formed amphiphile/DNA complexes. Additional discussion of the use of these ingredients in found in aforementioned WO 96/18372 at, for example, page 87.

pCFI-CFTR plasmid (containing an encoding sequence for human cystic fibrosis transmembrane conductance regulator, see Example 4) is provided in water-for-injection at a concentration, measured as nucleotide, of 4 mM. Complexing of the plasmid and amphiphile is then allowed to proceed by gentle contacting of the two solutions for a period of 10 minutes.

It is presently preferred to deliver aerosolized DNA to the lung at a concentration thereof of between about 2 and about 12 mM (as nucleotide). A sample of about 10 to about 40 ml is generally sufficient for one aerosol administration to the lung of an adult patient who is homozygous for the Δ F508 mutation in the CFTR-encoding gene.

It is expected that this procedure (using a freshly prepared sample of amphiphile/DNA) will need to be repeated at time intervals of about two weeks, but depending considerably upon the response of the patient, duration of expression from the transfected DNA, and the appearance of any potential adverse effects such as inflammation, all of which can be determined for each individual patient and taken into account by the patient's physicians.

One important advantage of the cationic amphiphiles of the present invention is that they are substantially more effective—in vivo—than other presently available amphiphiles, and thus may be used at substantially lower concentrations than

known cationic amphiphiles. There results the opportunity to substantially minimize side effects (such as amphiphile toxicity, inflammatory response) that would otherwise affect adversely the success of the gene therapy.

A further particular advantage associated with use of many of the amphiphiles of the invention should again be mentioned. Many of the amphiphiles of the invention were designed so that the metabolism thereof would rapidly proceed toward relatively harmless biologically-compatible components.

Example 5-Alternate Procedure to Prepare an Amphiphile /Co-lipid Composition

In order to formulate material that is suitable for clinical administration, it may be preferable to avoid use of chloroform when the cationic amphiphile and the co-lipid are prepared together. An alternate method to produce such compositions may be as follows.

The cationic amphiphile, the neutral co-lipid DOPE, and $PEG_{(5000)}$-DMPE are weighed into vials, and each is dissolved in t-butanol:water 9:1 with vortexing, followed by transfer to a single volumetric flask. An appropriate amount of each lipid is selected to obtain a molar ratio of cationic amphiphile to DOPE to DMPE-PEG of 1:2: 0.05. The resultant solution is then vortexed, and further diluted as needed with t-butanol:water 9:1, to obtain the desired concentration. The solution is then filtered using a sterile filter (0.2 micron, nylon).

One mL of the resultant filtered 1:2: 0.05 solution is then pipetted into individual vials. The vials are partially stoppered with 2-leg butyl stoppers and placed on a tray for lyophilization. The t-butanol:water 9:1 solution is removed by

freeze drying over 2 to 4 days at a temperature of approximately -5°C. The lyophilizer is then backfilled with argon that is passed through a sterile 0.2 micron filter. The stoppers are then fully inserted into the vials, and the vials are then crimped shut with an aluminum crimp-top. The vials are then maintained at -70°C until use.

An alternate lyophilization procedure is as follows. 4 mL of stock solution (amphiphile No. 67: DOPE: PEG ($_{5000}$)-DMPE as 1:2: 0.05) in tBuOH:dH$_2$O (9:1) was placed in a 20 mL serum vial and partially stoppered with a 2-leg butyl stopper. The vials were placed on a precooled shelf (-30 degrees C) in a tray lyophilizer and allowed to cool for 30 minutes. Vacuum was applied and the samples were held at approximately 200 mTorr for 1 hour. The shelf temperature was then raised to -5 degrees C for 8 hours, and then the shelf temperature was raised to 20 degrees C. The samples were then allowed to lyophilize to dryness (about 16 hours). The lyophilizer was backfilled with filtered argon gas and the samples were stoppered. The vials were sealed with an aluminum crimp top and stored at -80 degrees C.

We claim:

1.   A composition comprising:

    1) a cationic amphiphile;

    2) a biologically active molecule;

    3) a PEG-derivative; and optionally

    4) a co-lipid,

wherein said PEG derivative is a PEG polymer having a molecular weight ranging

from about 1,000 to 10,000 with a dilaurylphosphatidylethanolamine (DLPE) group

attached to said PEG polymer.

2.   A composition according to Claim 1 wherein said PEG-derivative is chosen

from PEG_{2000}-DLPE or PEG_{5000}-DLPE.

3.   A composition according to Claim 1 wherein the molar ratio therein of said

cationic amphiphile to PEG-derivative is 1: 0.125.

4.   A composition according to Claim 1 wherein the molar ratio therein of said

cationic amphiphile to co-lipid is 1:2 , and the molar ratio of said cationic amphiphile

to PEG-derivative is 1: 0.125.

5.   A composition comprising:

    1) a cationic amphiphile;

2) a biologically active molecule;

3) a PEG-derivative; and

4) a co-lipid, wherein said co-lipid is diphytanoylphosphatidylethanolamine.

6.   A composition according to Claim 5 wherein said PEG derivative is a PEG polymer with a hydrophobic group attached to said PEG polymer.

7.   A composition according to Claim 6 wherein the molecular weight of said PEG polymer ranges from about 1,000 to 10,000.

8.   A composition according to Claim 6 wherein said PEG derivative is selected from PEG-DMPE, PEG-DOPE, PEG-DPPE, PEG-DSPE and PEG-DLPE and wherein the PEG polymer group of said PEG derivative has a molecular weight ranging from about 1,000 to 10,000 .

9.   A composition according to Claim 5 wherein said PEG derivative is selected from PEG_{2000}-DMPE, PEG_{5000}-DMPE, PEG_{2000}-DOPE, PEG_{5000}-DOPE, PEG_{2000}-DPPE, PEG_{5000}-DPPE, PEG_{2000}-DSPE, PEG_{5000}-DSPE, PEG_{2000}-DLPE, and PEG_{5000}-DLPE.

10.   A composition according to Claim 5 wherein the molar ratio therein of said cationic amphiphile to co-lipid is 1:2 , and the molar ratio of said cationic amphiphile to PEG-derivative is 1: 0.125.

11. A composition according to Claim 5 wherein the molar ratio therein of said cationic amphiphile to PEG-derivative is 1: 0.125.

12. A method of delivering a biologically active molecule to a mammalian cell comprising the step of administering to a mammal a composition comprising:

    1) a cationic amphiphile;

    2) a biologically active molecule;

    3) a PEG-derivative; and

    4) a co-lipid, wherein said co-lipid is diphytanoylphosphatidylethanolamine.

13. A method according to Claim 12 wherein said PEG-derivative is selected from PEG_{2000}-DMPE, PEG_{5000}-DMPE, PEG_{2000}-DOPE, PEG_{5000}-DOPE, PEG_{2000}-DPPE, PEG_{5000}-DPPE, PEG_{2000}-DSPE, PEG_{5000}-DSPE, PEG_{2000}-DLPE, and PEG_{5000}-DLPE.

14. A method of transfecting a gene in a mammalian cell comprising the step of administering to a mammal a composition comprising:

    1) a cationic amphiphile;

    2) a biologically active molecule;

    3) a PEG-derivative; and

    4) a co-lipid, wherein said co-lipid is diphytanoylphosphatidylethanolamine.

15. A method according to Claim 14 wherein said PEG-derivative is selected from

PEG_{2000}-DMPE, PEG_{5000}-DMPE, PEG_{2000}-DOPE, PEG_{5000}-DOPE, PEG_{2000}-DPPE,

PEG_{5000}-DPPE, PEG_{2000}-DSPE, PEG_{5000}-DSPE, PEG_{2000}-DLPE, and PEG_{5000}-DLPE.

1/1

DMPE

FIG. 1